

# Light-induced activation of the rod phosphodiesterase leads to a rapid transient increase of near-infrared light scattering

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The so-called AT-signal described here is a transient light-induced increase of the near-infrared scattering from isolated bovine rod outer segments (ROS). Freshly prepared ROS are permeabilized with 0.01% Triton X-100 immediately before measurement in the presence of 1 mM GTP. The signal amplitude is saturated when ~2 rhodopsin molecules out of 30 000 are photo-excited. The signal recovers rapidly (~90 s) and can be repeated in a succession of flashes. The AT-signal can be prevented by pre-activation of the phosphodiesterase (PDE) enzyme cascade at various levels: either at the level of G-protein, using  $ALF_4^-$  in darkness or GTP $\gamma$ S plus light; or at the level of the PDE catalytic unit, using protamine as an activator. The light sensitivity and kinetics of the AT-signal are similar to published parameters of PDE activation. These data suggest that light-induced activation of the PDE is the key reaction for the generation of the signal. On the other hand, blocking of the catalytic cGMP binding site by isobutylmethylxanthine only slightly affects the signal. We propose that the AT-signal reflects a structural change linked to the transient removal of the PDE inhibitory subunit from the catalytic unit.

*Rod outer segment      Rhodopsin      Time-resolved light scattering      Phosphodiesterase*

## 1. INTRODUCTION

Two membrane systems are implicated in the signal transduction of the rod photoreceptor: the stack of disc membranes that carry the visual pigment rhodopsin together with amplifier proteins and the plasma membrane providing the site where the electrical response is generated.

Membranes and interconnecting structures [1,2] constitute the highly ordered rod outer segment (ROS). Time-resolved light scattering has revealed characteristic light-induced changes of the ROS

structure [3,4]. More recently these so-called light scattering signals have been recognized as monitors of membrane-bound biochemical reactions [5]. The signals studied so far have been attributed to the light-induced interaction between rhodopsin and G-protein at the disc membrane surface [5–8] and to an  $Mg^{2+}$ -ATPase activity of the disc membrane [9].

Fig.1 presents a scheme of the G-protein reaction pathway which leads to rod PDE activation. Light scattering monitors are available for stages I–III: for stage I the N-signal or rhodopsin signal [3,5], for stage II the P-signal or binding signal [5,6], and for stage III the dissociation, loss and release signals [5,10] and the so-called  $G^+$  signal [7] which represent different physical monitors of G-protein activation.

Here we introduce a monitor for stage IV, the so-called AT-signal. This signal is measured on structurally intact ROS and seems to follow the switch-on and -off of the PDE rather closely.

**Abbreviations:** NIR, near infrared; PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; GTP $\gamma$ S, guanosine 5'-O-(3-thiotriphosphate); G, G-protein (or transducin);  $G_{GTP}$ , G-protein in the GTP-binding form

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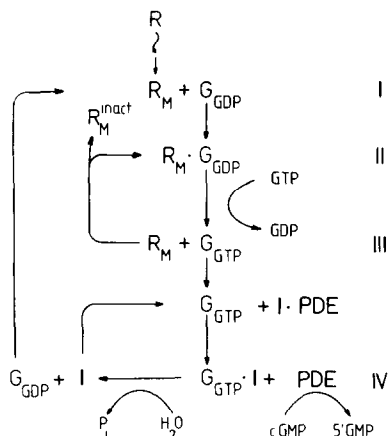


Fig.1. Simplified scheme (cf. [5,13,15,17]) of the reaction steps leading to activation of the rod PDE. I-IV denote the stages where light scattering signals are measured. Photo-excitation of rhodopsin (R) leads to the conformation metarhodopsin II ( $R_M$ ) which binds to  $G_{DDP}$ .  $R_M \cdot G_{DDP}$  interaction enables GTP/GDP exchange which dissociates.  $R_M \cdot G$ , recycling  $R_M$  and producing free  $G_{GTP}$ . This species activates (de-inhibits) the PDE by interaction with the inhibitor I. GTP-ase activity of  $G_I$  leads to  $G-I$  dissociation and recycling of  $G_{DDP}$ .

## 2. MATERIALS AND METHODS

GTP was supplied by Serva Heidelberg.  $GTP\gamma S$  was from Boehringer, Mannheim. Protamine sulfate (grade X) were from Sigma, Munich. IBMX was from Aldrich. All chemicals were of the highest purity available.

For this study we used the bovine preparation by Schnetkamp et al. with some modifications [11]. Fresh bovine retinae were dissected under dim red light ( $>650$  nm) and kept at  $10^\circ\text{C}$  for 20 min in 5 mM Tris-acetate buffer (pH 7.6), 10 mM glucose, 1 mM  $\text{CaCl}_2$ , 2 mM dithiothreitol (DTT), 0.1 mM EDTA, 660 mM sucrose. After isolating the outer segments on a continuous sucrose gradient (23–36%, w/w, sucrose), made up in 2 mM Tris-acetate and 1 mM  $\text{Mg}(\text{OAc})_2$ , pH 7.6, the ROS suspension was kept in this medium at  $0^\circ\text{C}$ . This preparation was sealed as indicated by the low extractable amounts of G-protein.

Isolated outer segments were permeabilized was performed by 0.008% Triton X-100, added immediately before NIR light scattering measurement in a medium offering approximately intracellular

conditions: 70 mM KCl, 40 mM  $\text{NaHCO}_3$ , 5 mM  $\text{Mg}(\text{OAc})_2$ , 0.5 mM  $\text{KH}_2\text{PO}_4$ , 1 mM DTT (pH 7.6). This medium was supplied with 0.5–1.0 mM GTP. The pH was buffered to 7.6 with 3 mM Hepes and Tris. The free  $\text{Ca}^{2+}$  concentrations were in the micromolar range. Additional free  $\text{Ca}^{2+}$  levels up to  $10^{-5}$  M did not significantly reduce the signal amplitude nor its rate. The Triton X-100 concentration of 0.008% (v/v) is about half the critical micelle concentration (CMC) under our conditions (cf. [12]).

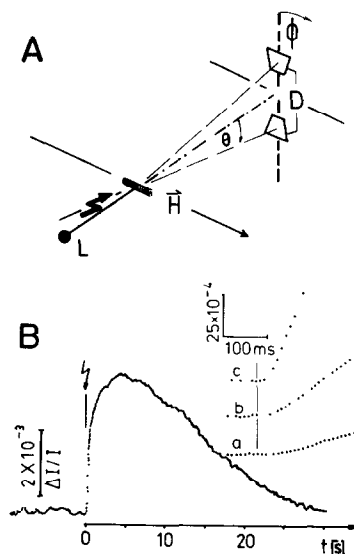


Fig.2. Measurement of the amplified NIR scattering transient (AT-signal) on isolated rod outer segments. (A) Optical setup: L, near-infrared light emitting diode (850 nm) and condenser; H, magnetic field, standard 8 kG; D, detector consisting of two photovoltaic diode arrays. The ROS are axially ordered by the magnetic field. Symbolically one outer segment is shown.  $\phi$  indicates the azimuthal orientation of the detector D relative to the ROS axis. L yields a steady incident beam, normal to the ROS axis. The sensitive areas of D are rotationally symmetric to the incident beam. D is shown in its standard orientation (used throughout this study), normal to the plane of ROS axis and optical axis. Its sensitive areas cover the range  $\theta = 25\text{--}30^\circ$  and  $\phi = -10$  to  $+10^\circ$ . (B) AT-signal. The trace is from the first flash on a fresh sample of permeabilized ROS (see methods) measured at  $30^\circ\text{C}$  and pH 7.6. The flash photo-excited a  $1.5 \times 10^{-4}$  mol fraction of rhodopsin. Inset: Early phase of the AT-signal for 3 different flash intensities, given in mol fractions of photo-excited rhodopsin, (a)  $3.75 \times 10^{-5}$ , (b)  $1.5 \times 10^{-4}$ , (c)  $7.5 \times 10^{-4}$ . The temperature with these experiments was  $22^\circ\text{C}$ , pH = 7.6.

We oriented the ROS magnetically and measured the NIR scattering by detector arrays [4]. All measurements were performed within 2 h after preparation of the outer segments; within this time, the decrease of the scattering anisotropy (see below) is slow. The sample volume was 2 ml; rhodopsin concentration was  $1 \mu\text{M}$ .

The optimal geometry for measuring the flash-induced scattering change (applied throughout this study) was  $\theta = 25 - 30^\circ$ ,  $\phi = 0^\circ$  (normal to the

plane given by ROS axis and optical axis, see fig. 2).

In the apparatus shown in fig. 2A, orientation of the bovine ROS in the 8 kG magnetic field proceeds with a half mean time of 3 s and leads after  $\sim 30$  s to a stable anisotropic scattering pattern. The radial scattering of the ROS ( $\phi = 0$ ) was typically 4-times higher than the axial scattering ( $\phi = 90^\circ$ ) reflecting the geometry and state of order of the ROS [4].

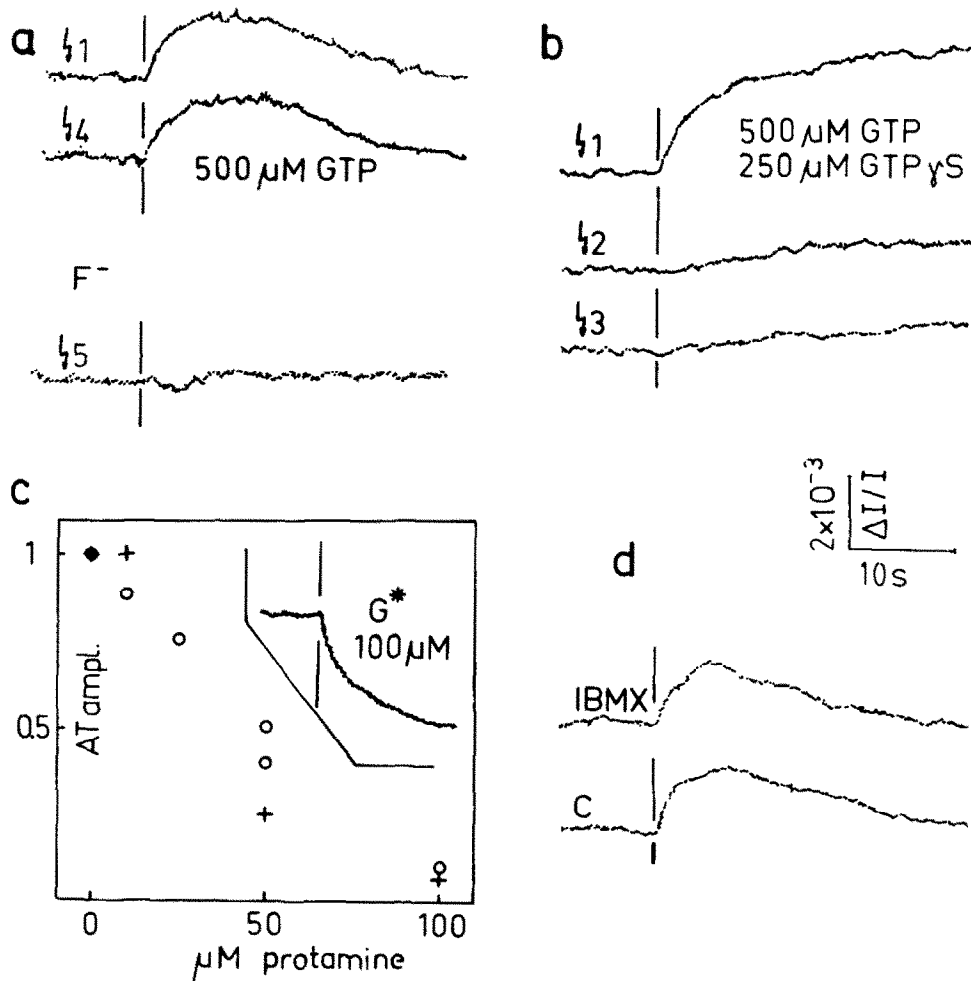


Fig.3. Tests of the AT-signal on different stages (fig.1) of the cGMP-cascade. (a) AT-signals from flash nos 1 and 4 in a succession of flashes evoking equal signals in 90 s intervals; flash no. 5 was given after application of  $0.1 \text{ mM A1F}_4^-$ ; (b) Lack of the negative component  $\text{AT}^-$  and of recovery in a succession of flashes in the presence of additional  $250 \mu\text{M GTP}\gamma\text{S}$ . (c) Relative amplitude of the AT-signal after treatment (3 min) of permeabilized ROS samples with protamine in the concentrations given on the abscissa. The inset shows the dissociation signal (cf. [5]) after fragmentation of the ROS and in the presence of  $100 \mu\text{M}$  protamine. (d) AT-signals after incubation of the sample (20 min at  $25^\circ\text{C}$ ) in  $1 \text{ mM IBMX}$ . C denotes the control sample incubated without IBMX. Measuring parameters:  $T = 22^\circ\text{C}$ ,  $\text{pH} = 7.6$ , mol fraction of photo-excited rhodopsin  $1.5 \times 10^{-4}$  (a,b,d) and  $7.5 \times 10^{-4}$  (c).

### 3. RESULTS

Isolated bovine ROS [11] were permeabilized and suspended in the measuring buffer (see section 2). The static anisotropy of the permeabilized ROS in this medium does not deviate from that of the original ROS.

The track in fig.2B shows the flash-induced transient change of the scattering intensity measured at 30°C and at pH 7.6. The flash photo-excited a rhodopsin mol fraction of  $10^{-4}$ , i.e.  $\sim 3$  molecules per disc membrane. Detectable signals are already evoked by a rhodopsin turnover of  $5 \times 10^{-6}$ . This high light sensitivity certainly involves amplification and, therefore, we term this signal the 'amplified transient' and use the abbreviation 'AT-signal'.  $AT^+$  and  $AT^-$  denote its positive and negative component, respectively.

The only well-documented amplification process in ROS is the rhodopsin-/G-protein/phosphodiesterase (R/G/PDE) enzymatic cascade ([13]; fig.1). The experiments presented in fig.3 test various levels of the cascade for their contribution to the AT-signal. In fig.3a, the first two AT-signals are representative of a series of equal observations evoked by a succession of flashes. A series of 10–20 AT-signals can be evoked with the most stable samples. In many samples the  $AT^-$  component of the signals from the first (and often from the second) flash is smaller or slower.  $AT^+$  and  $AT^-$  normally balance after some flashes and stable transients are evoked by further flashes.

$ALF_4^-$  can be used to test the G-protein for its role in the generation of the AT-signal. This agent has an effect on the G-protein [14] which leads to activation of the PDE and should, therefore, prevent subsequent light-induced activation. As fig.3a shows, addition of 0.1 mM  $ALF_4^-$  to the ROS suspension after flash no. 4 prevents the light-induced AT-signal from flash no. 5 (and further flashes). An additional test for the G-protein level is given by the non-hydrolysable GTP-analog  $GTP\gamma S$  which keeps the G-protein persistently light-activated (in the  $G_{GTP}$  form) thereby also preventing subsequent light activation. In the presence of  $GTP\gamma S$  (fig.3b) after 1–2 flashes, no AT-signal can be observed any longer. These data provide evidence that the light activation of G-protein is an essential event in the generation of the AT-signal.

To test the requirement for PDE activity in the generation of the AT-signal, we attempted to activate the ROS phosphodiesterase by an agent that does not affect the R and G stages. Protamine is known to activate the PDE [15]. We first verified that this agent has no indirect effect on the G-protein activation (stage III). Treatment of ROS fragments with 100  $\mu M$  protamine allows a normal dissociation signal (fig.3c, inset) demonstrating undisturbed G-protein activation [5]. As fig.3c shows, pretreatment of ROS with protamine markedly reduces the AT-signal; pre-activation by 100  $\mu M$  protamine completely prevents the AT-signal. This finding provides strong evidence that light-induced PDE activation (stage IV) parallels the AT induction.

Because PDE catalyses the hydrolytic breakdown of cGMP to 5'-GMP, the question arises as to whether the AT-signal is mediated by cGMP hydrolysis. A well-known test for this reaction is its inhibition by IBMX which binds to the catalytic binding site for cGMP. Strikingly, even 1 mM IBMX does not significantly influence the AT-signal (fig.3d). Thus, a trigger role for cGMP

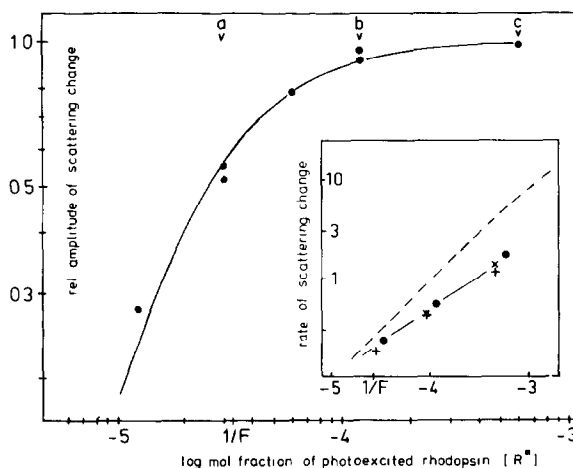


Fig.4. Saturation of the AT-signal with the mol fraction of photo-excited rhodopsin ( $R^*$ ) in one flash. The AT amplitude (—) reaches 60% of its maximum at  $R^* = 3.3 \times 10^{-5} = 1/F$ .  $F$  is 30 000, i.e. the number of rhodopsin molecules in one disc membrane. The inset shows the rate of rise of the AT-signal as a function of  $R^*$ , compared to the rate of G-activation (---, taken from [10]). See text for details. Different symbols are different preparations; temperature 22°C, pH = 7.6.

Meaning of a–c as in fig. 1B.

hydrolysis in the induction or generation of the AT-signal can be excluded.

The crucial event for the AT-signal apparently is the de-inhibition of the PDE (stage IV in fig.1). Because the PDE is activated by stoichiometric binding of G to the PDE inhibitor (PDE·I) and because much less PDE than G-protein is present in the ROS one would expect that the AT-signal saturates at lower light intensities than the dissociation signal. This is really observed as shown in fig.4. The saturation is represented as a function of the relative mol fraction of R\* (left curve). It is seen that the AT-peak reaches 60% of its maximal value for a relative rhodopsin turnover  $1/F = 3.3 \times 10^{-5}$ , i.e. when one rhodopsin molecule out of 30 000 is photo-excited. This corresponds to the number of rhodopsin molecules in one disc membrane (one side of a disc).

The inset of fig.4 is a plot of the AT-rate vs R\*. Following [10], the rate is defined as the maximal slope of the scattering increase (% scattering change per s) divided by the maximal amplitude under saturating conditions (% scattering change). The dashed line is directly taken from [10] and reflects the rate of G-protein activation in frog ROS (so-called release signal). The rate of AT<sup>+</sup> is reduced with increasing R\* as compared to G-activation whereby the signals at different R\* are mathematically similar and fit reasonably to each other by scaling (fig.2b, inset).

#### 4. DISCUSSION

A novel light-induced NIR scattering signal (AT-signal) from bovine ROS has been described.

The signal is saturated with flashes photo-exciting a rhodopsin mol fraction as low as  $10^{-4}$  of the visual pigment rhodopsin (R) and requires structurally preserved ROS. These are conditions where the other known signals are far from saturation and/or physically impossible (cf. [5–8] and section 1). We have provided evidence that all the steps in the cGMP cascade of vision (fig.1) including activation of the phosphodiesterase (PDE) are necessary for the generation of the signal (fig.3). The catalytic activity of the PDE is apparently not required because even 1 mM IBMX does not considerably affect the AT-signal. Therefore, this light scattering signal could be explained by the de-inhibition of the PDE, the

removal of the inhibitor I from the PDE·I complex (fig.1). This reaction might well involve major protein displacement and might be directly linked to a change of structure observable in light scattering. Moreover, this concept would fit to the other scattering signals which are known to arise from different stages (fig.1) of the G-protein pathway. The relaxation of rhodopsin into the binding conformation for the G-protein (stage I), the binding of G to R (II), the dissociation of G from R (III) and now, with the AT-signal, the reaction of G with I (IV) would be accessible and measurable in situ by kinetic light scattering.

The AT-signal can be compared with the PDE-activity monitor which measures the protons arising from cGMP hydrolysis [17]. Differentiation of the H<sup>+</sup> signals yields PDE activity transients [15] which are similar to the AT scattering signals. Both monitors are complementary to each other since the pH change is best measured on fragmented ROS while the AT-signal requires high structural integrity.

Fig.4 implies that photoactivation of the G-protein present in the ROS is able to saturate the AT-signal completely. In contrast, it has been reported [15] that saturation of the PDE activity in vitro requires an overloading of the membranes with a 4-fold amount of G-protein.

Not only the amplitude but also the rate of the AT-signal saturates (fig.4, inset). This occurs already when 2 out of 30 000 rhodopsin molecules in a disc membrane are simultaneously photo-excited. Because of the scaling of the signals at different intensities (fig.2B) the saturation appears to arise from a slowing of the R/G/PDE mechanism rather than from some postponed mechanism.

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